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GPER regulates endothelin-dependent vascular tone and intracellular calcium☆

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ABSTRACT

Aims: An increase in intracellular vascular smooth muscle cell calcium concentration (VSMC $[Ca^{2+}]_i$) is essential for endothelin-1 (ET-1)-induced vasoconstriction. Based on previous findings that activation of the G protein-coupled estrogen receptor (GPER) inhibits vasoconstriction in response to ET-1 and regulates $[Ca^{2+}]_i$ in cultured VSMC, we investigated whether endogenous GPER regulates ET-1-induced changes in VSMC $[Ca^{2+}]_i$ and constriction of intact arteries.

Main methods: Pressurized carotid arteries of GPER-deficient ($GPER^0$) and wildtype (WT) mice were loaded with the calcium indicator fura 2-AM. Arteries were stimulated with the GPER-selective agonist G-1 or solvent followed by exposure to ET-1. Changes in arterial diameter and VSMC $[Ca^{2+}]_i$ were recorded simultaneously. Vascular gene expression levels of ET_A and ET_B receptors were determined by qPCR.

Key findings: ET-1-dependent vasoconstriction was increased in arteries from $GPER^0$ compared to arteries from WT mice. Despite the more potent vasoconstriction to ET-1, GPER deficiency was associated with a marked reduction in the ET-1-stimulated VSMC $[Ca^{2+}]_i$ increase, suggesting an increase in myofilament force sensitivity to $[Ca^{2+}]_i$. Activation of GPER by G-1 had no effect on vasoconstriction or VSMC $[Ca^{2+}]_i$ responses to ET-1, and expression levels of ET_A or ET_B receptor were unaffected by GPER deficiency.

Significance: These results demonstrate that endogenous GPER inhibits ET-1-induced vasoconstriction, an effect that may be associated with reduced VSMC Ca^{2+} sensitivity. This represents a potential mechanism through which GPER could contribute to protective effects of endogenous estrogen in the cardiovascular system.

Introduction

Endothelin-1 (ET-1) is the most potent endogenous vasoconstrictor known (Yanagisawa et al., 1988) that regulates basal vascular tone, and contributes to the development of vascular diseases, such as arterial hypertension and atherosclerosis through stimulation of cell growth and inflammation (Barton and Yanagisawa, 2008). ET-1 is the predominant isoform of the endothelin peptide family, which acts in an autocrine and paracrine manner via G protein-coupled endothelin receptors ET_A and ET_B (Barton and Yanagisawa, 2008; Wynne et al., 2009). Whereas ET_A receptors mediate vasoconstriction and cell proliferation, ET_B receptors primarily facilitate the release of nitric oxide and ET-1 clearance (Barton and Yanagisawa, 2008; Wynne et al., 2009). In vascular smooth muscle cells (VSMC), ET_A receptor activation by ET-1 induces an increase in intracellular VSMC calcium concentration (VSMC $[Ca^{2+}]_i$), which is the intracellular messenger that triggers subsequent vasoconstriction (Wynne et al., 2009; Miwa et al., 2005).

Natural estrogens, such as 17β-estradiol, have been implicated in protection from cardiovascular diseases, including arterial hypertension and atherosclerosis (Meyer et al., 2006; Barton and Meyer, 2009). 17β-Estradiol inhibits ET-1-dependent vasoconstriction in several vascular beds (Jiang et al., 1992; Lamping and Nuno, 1996; Sudhir et al., 1997a; Teoh et al., 2000; Tsang et al., 2004a), possibly via inhibition of Ca^{2+} influx and/or stimulation of Ca^{2+} efflux in VSMC (Jiang et al., 1992; Prakash et al., 1999). 17β-Estradiol is a non-selective activator of estrogen receptors ERα and ERβ, which mainly function as ligand-activated transcription factors (Prossnitz and Barton, 2011). More recently, 17β-estradiol has also been shown to bind to and activate the G protein-coupled estrogen receptor GPER (previously termed GPR30), an intracellular seven-transmembrane spanning receptor (Revankar et al., 2005). GPER is expressed throughout the vascular system of humans and animals of both sexes (Prossnitz and Barton, 2011; Meyer et al., 2011), and animals lacking the GPER gene display increased endothelium-dependent contractility (Meyer et al., 2012). Moreover, the GPER-selective agonist G-1 (Bologa et al., 2006) induces acute vasodilation and indirectly inhibits the response to several vasoconstrictors including ET-1 (Prossnitz and Barton, 2011; Meyer et al., 2010; 2011; Haas et al., 2009; Broughton

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et al., 2010). Other GPER agonists such as selective estrogen receptor modulators (SERMs) including raloxifene, the selective estrogen receptor downregulator (SERD) fulvestrant, or the phytoestrogen genistein also inhibit vasoconstriction to ET-1 (Prossnitz and Barton, 2011; Meyer et al., 2010, 2011; Tsang et al., 2004b; Lee et al., 2004). Although selective GPER activation regulates $[Ca^{2+}]_i$ in VSMC cells (Haas et al., 2009) and other cell types (Prossnitz and Barton, 2011), it is unknown whether and to what extent endogenous GPER modulates the VSMC $[Ca^{2+}]_i$ response to vasoconstrictors such as ET-1 in intact arteries.

To test the hypothesis that endogenous GPER inhibits ET-1-induced arterial constriction by regulating changes in VSMC $[Ca^{2+}]_i$, we simultaneously measured changes in vascular tone and VSMC $[Ca^{2+}]_i$ in response to ET-1 in arteries from wildtype (WT) and GPER-deficient ($GPER^0$) mice. In addition, we determined the acute effects of selective GPER activation by G-1 on vascular responses.

Materials and methods

Materials

Fura 2-AM (fura 2-acetoxymethyl ester) and pluronic acid were from Invitrogen (Carlsbad, CA, USA), and ET-1 was from Enzo Life Sciences (Farmingdale, NY, USA). G-1 was synthesized as described (Bologa et al., 2006) (provided by Jeffery Arterburn, New Mexico State University, Las Cruces, NM, USA), and dissolved in ethanol. G-1 and ET-1 were diluted in bicarbonate buffered physiological saline solution (PSS, composition in mmol/L: 129.8 NaCl, 5.4 KCl, 0.83 $MgSO_4$, 0.43 NaH_2PO_4, 19 $NaHCO_3$, 1.8 $CaCl_2$, and 5.5 glucose) to the concentrations required before use.

Animals

Male C57Bl6 (3 months of age, The Jackson Laboratory, Bar Harbor, ME) and $GPER^0$ mice (Proctor & Gamble, Cincinnati, OH, provided by Jan S. Rosenbaum) were housed at the animal research facility of the University of New Mexico Health Sciences Center. $GPER^0$ mice were bred as described (Wang et al., 2008) and backcrossed 10 generations onto C57Bl6 mice. Animals were maintained under controlled temperature of 22–23 °C on a 12 h light, 12 h dark cycle and fed normal chow ad libitum. All procedures were approved by and carried out in accordance with institutional policies and the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isolated vessel preparation and experimental setup

Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (2.2 mg/g BW) and exsanguinated by cardiac puncture. Common carotid arteries were excised, immediately placed into ice-cold PSS and carefully dissected from adherent tissue. A subset of carotid arteries was snap-frozen in liquid nitrogen and stored at −80 °C until further analysis. For functional experiments, carotid arteries were transferred to a vessel chamber (Living Systems, St. Albans, VT, USA), cannulated on glass micropipettes at each end, and secured with silk ligatures. Arteries were pressurized to 90 mm Hg with PSS using a servo-controlled peristaltic pump (Living Systems), and superfused with PSS (37 °C, pH 7.4, oxygenated with 21% O_2, 5% CO_2, and balanced N_2) at a rate of 5 mL per minute as described (Allahdadi et al., 2008). To avoid flow-dependent changes in vessel diameter, experiments were performed with the distal cannula closed. Arterial segments that did not maintain pressure were discarded.

Measurement of carotid artery VSMC $[Ca^{2+}]_i$ and constriction

Following a 30 min equilibration period, pressurized arteries were incubated for 45 min with 2 μmol/L fura 2-AM and 0.05% pluronic acid in 4 mL HEPES-PSS (composition in mmol/L: 134 NaCl, 6 KCl, 1 $MgCl_2$, 10 HEPES, 2 $CaCl_2$, 0.026 EDTA, and 10 glucose; pH 7.4) in the dark at room temperature (Allahdadi et al., 2008). After incubation, arteries were washed with oxygenated PSS (37 °C) for 15 min to remove excess dye. Arteries were then stimulated with the GPER-selective agonist G-1 (3 μmol/L) (Bologa et al., 2006) or solvent (ethanol 0.1% vol/vol) for 20 min as described (Haas et al., 2009; Meyer et al., 2010), followed by exposure to ET-1 (10 nmol/L) as the constricting agonist. Some arteries were subsequently incubated in Ca^{2+} free PSS to determine maximal diameter, which did not differ from inner diameter before stimulation with ET-1 (data not shown). Fura 2-AM-loaded vessels were alternately excited at 340 nm and 380 nm at a frequency of 1 Hz with a HyperSwitch dual-excitation light source (IonOptix, Milton, MA, USA), and the respective 510 nm emissions collected with a photomultiplier tube (F_{340}/F_{380}). Background-subtracted F_{340}/F_{380} emission ratios as a measure of relative VSMC $[Ca^{2+}]_i$ and inner arterial diameter from bright-field images were continuously calculated with IonWizard software (Version 5.0, IonOptix) as described (Allahdadi et al., 2008). Vasoconstriction was calculated as percent reduction of the maximal internal arterial diameter.

Quantitative real-time polymerase chain reaction (qPCR)

Frozen carotid arteries were disrupted and homogenized using a rotor-stator homogenizer, and total RNA was extracted using the silica-based RNeasy Fibrous Tissue Mini Kit (Qiagen, Valencia, CA, USA). RNA was reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). qPCR was performed using TaqMan gene expression assays on a 7500 FAST real-time PCR System (Applied Biosystems). The following sets of primers were used: 5′-GAAGGACTGGTGGCTCTTTG-3′ (forward) and 5′-CTTCTCGACGCTGTTTGAGG-3′ (reverse) for amplification of a specific cDNA fragment encoding for murine ET_A receptor (GenBank ID: BC008277); 5′-CGGTATGCAGATTGCTTTGA-3′ (forward) and 5′-CACCTGTGTGGATTGCTCTG-3′ (reverse) for amplification of a specific cDNA fragment encoding for murine ET_B receptor (GenBank ID: BC026553); 5′-TTCACCACCATGGAGAAGGC-3′ (forward) and 5′-GGCATGGACTGTGGTCATGA-3′ (reverse) for amplification of a specific cDNA fragment encoding for murine GAPDH (GenBank ID: NM_008084), which served as house-keeping control. Gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

Statistical analyses

Data was analyzed using the Student's *t*-test or two-way repeated-measures ANOVA followed by Bonferroni post hoc analysis where appropriate (Prism version 5.0 for Macintosh, GraphPad Software, San Diego, CA, USA). Values are shown as mean ± SEM of independent experiments. A $P<0.05$ value was considered statistically significant.

Results

Effect of GPER deficiency on ET-1-induced vasoconstriction

The vasoconstrictor response to ET-1 was significantly greater in arteries from $GPER^0$ mice (31.8 ± 1.5% vs. 24.0 ± 2.9%, $n=5$, $P<0.05$ vs. WT; Figs. 1 and 2). However, the kinetics of vasoconstriction, measured as the time to maximal constriction, were delayed by approximately 90 s in $GPER^0$ arteries (506 ± 26 vs. 413 ± 22 s, $n=5$, $P<0.03$ vs. WT; Fig. 2C). Acute selective GPER activation by G-1 had no effect on ET-1-induced vasoconstriction (26.7 ± 1.6% vs. 24.0 ± 2.9%, $n=5$–7, $P=$ n.s. vs. solvent).

GPER-dependent regulation of ET-1-stimulated VSMC $[Ca^{2+}]_i$ increase

Despite the greater vasoconstrictor response to ET-1, GPER deficiency was associated with a 40% reduction in the ET-1-stimulated VSMC $[Ca^{2+}]_i$ increase (0.097 ± 0.015 vs. 0.162 ± 0.018, $n=7$–8, $P<0.02$ vs. WT; Figs. 1 and 3A). Steady-state VSMC $[Ca^{2+}]_i$ did not

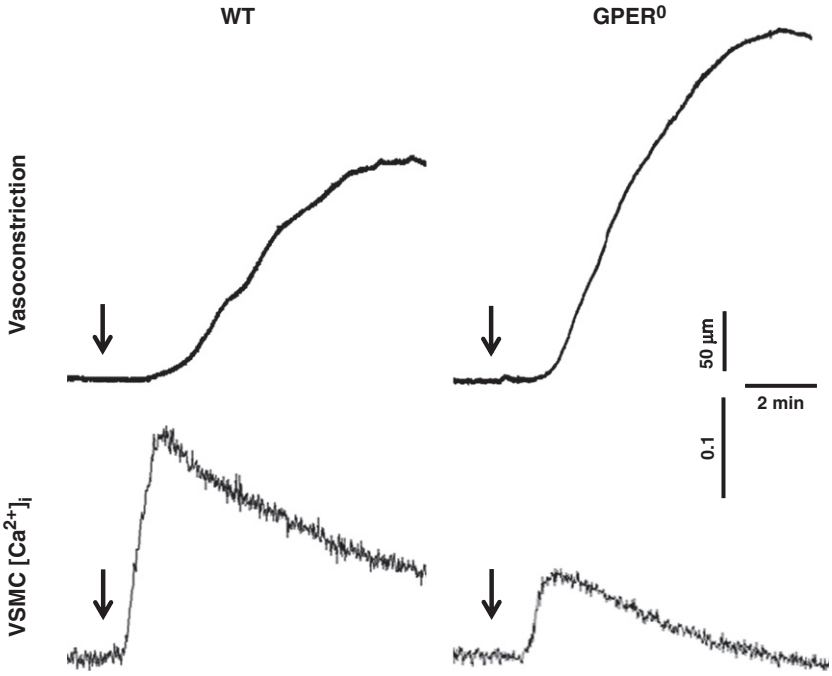

Fig. 1. Original recordings of vasoconstrictor responses (measured as change in arterial diameter, top) and VSMC $[Ca^{2+}]_i$ (given as relative F_{340}/F_{380} ratio, bottom) of carotid arteries from WT and $GPER^0$ mice following exposure to endothelin-1 (10 nmol/L, arrows). Changes in arterial diameter and VSMC $[Ca^{2+}]_i$ were recorded simultaneously.

differ between WT and $GPER^0$ mice (0.579 ± 0.012 vs. 0.561 ± 0.008, $n = 7$–8, P = n.s., Fig. 3A). Moreover, acute selective GPER activation by G-1 had no effect on the calcium response evoked by ET-1 in WT arteries (0.749 ± 0.039 vs. 0.740 ± 0.021, $n = 7$, P = n.s. vs. solvent, Fig. 3A). In addition to the overall inhibition of ET-1-stimulated VSMC $[Ca^{2+}]_i$, time-dependent VSMC $[Ca^{2+}]_i$ responses were delayed 1.4-fold in $GPER^0$ arteries independent of treatment with G-1 or solvent (66.7 ± 6.8 vs. 48.6 ± 2.4 s for maximal VSMC $[Ca^{2+}]_i$ increase, $n = 14$, $P < 0.02$ vs. WT; Fig. 3B).

Effect of GPER deficiency on endothelin receptor expression

In view of the greater vasoconstrictor response to ET-1 in GPER deficient animals, we evaluated steady-state ET_A and ET_B receptor mRNA expression levels in carotid arteries of WT and $GPER^0$ mice. GPER deficiency affected neither vascular gene expression of ET_A receptor nor ET_B receptor (Table 1), indicating that the more potent response to ET-1 in $GPER^0$ mice is not due to changes in receptor expression levels. Moreover, vascular gene expression levels of ET_A receptor were 5.7-fold higher than ET_B receptor in arteries of both WT and GPER0 mice ($P \leq 0.001$; Table 1).

Discussion

This study has identified endogenous GPER as a regulator of ET-1-induced vasoconstriction and the associated changes in VSMC $[Ca^{2+}]_i$ in intact arteries. GPER deficiency results in increased contractility despite a pronounced reduction in ET-1-stimulated VSMC $[Ca^{2+}]_i$, suggesting an increase in myofilament force sensitivity to $[Ca^{2+}]_i$ in arteries lacking GPER.

We have previously reported that the GPER agonists G-1 and ICI 182,780 acutely inhibit isometric contractions to ET-1 in porcine coronary arteries (Meyer et al., 2010). The present study extends these findings showing that in murine carotid arteries, the presence of endogenous GPER is sufficient to inhibit vasoconstriction to ET-1 without affecting ET_A or ET_B receptor gene expression levels. Similarly, we recently reported that GPER deletion increases endothelium-dependent contractility (Meyer et al., 2012). In carotid arteries used in the present study, the GPER-selective agonist G-1 had no acute effect on ET-1-induced constriction, indicating differential effects of chronic basal activity and acute activation of GPER. Moreover, differences in responsiveness to G-1 between species and vascular beds may explain its varied effects on ET-1-induced contraction in murine carotid and porcine coronary arteries (Meyer et al., 2010). Similarly, while estrogen acutely inhibits vasoconstriction to ET-1 in several vascular beds of different species (Jiang et al., 1992; Lamping and Nuno, 1996; Sudhir et al., 1997a; Teoh et al., 2000), this effect might not apply to all arteries (Haas et al., 2007).

We have previously shown in isolated VSMC that direct administration of the GPER-selective agonist G-1 acutely increases $[Ca^{2+}]_i$, while pretreatment with G-1 inhibits serotonin-mediated Ca^{2+} increases (Haas et al., 2009). Moreover, it has been demonstrated that the non-selective ER agonist 17β-estradiol acutely inhibits ET-1-stimulated $[Ca^{2+}]_i$ increases in coronary artery VSMC from female pigs by stimulating Ca^{2+} efflux (Prakash et al., 1999). The present study now demonstrates that GPER modulates ET-1-stimulated VSMC $[Ca^{2+}]_i$ in intact arteries providing further evidence that endogenous GPER is specifically involved in the regulation of VSMC $[Ca^{2+}]_i$ and the subsequent constrictor response to ET-1.

Ca^{2+} is a principal intracellular messenger that triggers cross-bridge cycling between actin filaments and myosin resulting in VSMC contraction (Jackson, 2000). However, vasoconstrictors such as ET-1 may increase myosin regulatory light chain (MLC) phosphorylation and VSMC force under certain conditions while $[Ca^{2+}]_i$ is clamped, a phenomenon referred to as Ca^{2+} sensitization (Wynne et al., 2009; Somlyo and Somlyo, 2003). Our observation that GPER deficiency is associated with greater ET-1-induced vasoconstriction despite a markedly reduced increase in VSMC $[Ca^{2+}]_i$ suggests an increase in myofilament force sensitivity to even low levels of $[Ca^{2+}]_i$. Interestingly, the lack of GPER considerably delayed the contractile response to ET-1, which could reflect the time required for activation of pathways involved in regulating MLC phosphorylation (Somlyo and Somlyo, 2003). Endogenous estrogens have previously been implicated in reducing the sensitivity of contractile proteins to Ca^{2+}: In basilar arteries of male and ovariectomized female rats, reducing MLC phosphorylation by inhibition of Rho-associated kinase (ROK) results in a stronger dilator response compared to females with intact

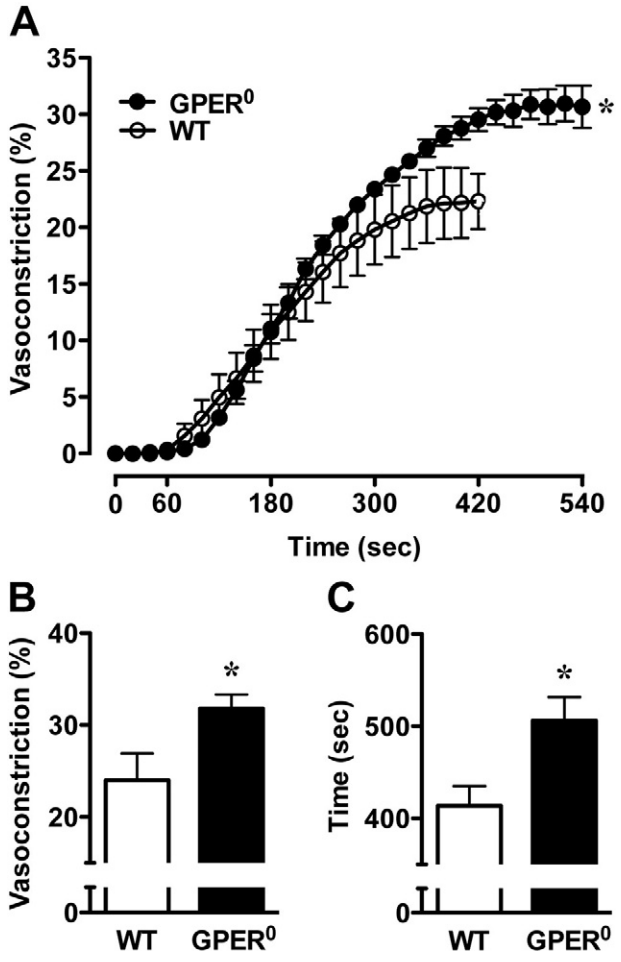

Fig. 2. Endothelin-1-induced constriction in carotid arteries of WT and $GPER^0$ mice. Time-dependent vasoconstriction to endothelin-1 (10 nmol/L, A), maximal responses (B), and time until maximal constriction (C) are shown. Vasoconstriction was calculated as the percent reduction of the maximal internal arterial diameter. Values are means ± SEM; $n = 5$. *$P<0.05$ vs. WT.

ovaries or receiving 17β-estradiol replacement (Chrissobolis et al., 2004). Moreover, greater serotonin-dependent contractions in aorta from male compared to female mice are abolished after ROK inhibition (Nuno et al., 2007). Based on the recognition of GPER as a potential mediator of the Ca^{2+} desensitizing effects of estrogens, future studies should address whether and through which mechanisms GPER regulates MLC phosphorylation independent of changes in VSMC $[Ca^{2+}]_i$.

The present study was performed in arteries from male mice, indicating that GPER, a receptor for a hormone traditionally implicated in female biology, also affects the male vasculature. Indeed, GPER is expressed in the cardiovascular system of both females and males (Prossnitz and Barton, 2011; Meyer et al., 2011), with GPER-mediated acute relaxation of carotid arteries observed in male rodents (Haas et al., 2009; Broughton et al., 2010). However, basal intrinsic activity of GPER in the absence of a ligand, which has been observed for many other G protein-coupled receptors (Gilchrist and Blackmer, 2007), may be sufficient to mediate vascular effects. Furthermore, local conversion from androgen precursors into estrogens by the enzyme aromatase within the vascular wall could yield sufficient tissue concentrations to mediate relevant physiological effects (Prossnitz and Barton, 2011; Meyer et al., 2011). In addition, genetic deletion of the classical ERα and ERβ has adverse effects on vascular tone in males (Rubanyi et al., 1997; Sudhir et al., 1997b; Zhu et al., 2002). Taken together, these findings indicate a role for multiple estrogen receptors in male vascular physiology. Nevertheless, future studies should address potential sex differences in GPER-dependent regulation of ET-1-induced VSMC $[Ca^{2+}]_i$ and vasoconstriction.

Table 1
Gene expression levels of ET_A and ET_B receptors in carotid arteries of WT and $GPER^0$ mice. Values are calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) and expressed as arbitrary units (mean ± SEM); $n = 5–6$.

Gene	WT	$GPER^0$
ET_A receptor	304 ± 54	336 ± 18
ET_B receptor	53 ± 9*	59 ± 13*

* $P<0.001$ vs. ET_A receptor.

Conclusions

GPER-mediated inhibition of the ET-1-stimulated VSMC $[Ca^{2+}]_i$ increases represents a newly identified mechanism that may mediate the observed inhibition of ET-1-dependent vasoconstriction. Tonic inhibition of ET-1-induced vasoconstriction by endogenous GPER may contribute to the protective effects of estrogens or other GPER agonists such as SERMs (Prossnitz and Barton, 2011; Meyer et al., 2011) in the pathogenesis of cardiovascular diseases (Meyer et al., 2006; Barton and Meyer, 2009).

Conflict of interest statement

E.R.P. is an inventor on United States Patent Number 7,875,721.

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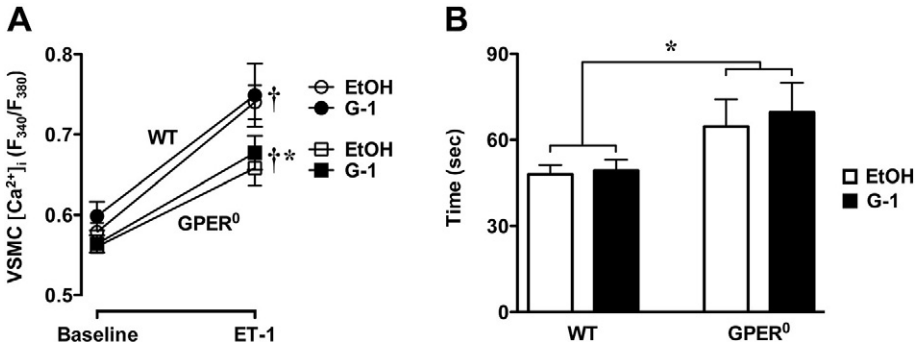

Fig. 3. Changes in VSMC $[Ca^{2+}]_i$ (A) and time to maximal VSMC $[Ca^{2+}]_i$ increase (B) in response to endothelin-1 (ET-1, 10 nmol/L). Carotid arteries of WT and $GPER^0$ mice were pretreated with the GPER-selective agonist G-1 (3 μmol/L) or solvent (ethanol, EtOH 0.1% vol/vol) for 20 min. Values are means ± SEM; $n = 6–8$. *$P<0.05$ vs. WT; †$P<0.05$ vs. baseline.

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